From the DEPARTMENT OF MEDICINE, HUDDINGE
Karolinska Institutet, Stockholm, Sweden

MODELS OF THE LUNG TISSUE MICROENVIRONMENT FOR STUDIES OF HUMEN MYELOID CELL FUNCTION

Puran Chen

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Models of the lung tissue microenvironment for studies of human myeloid cell function

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Puran Chen

Principal Supervisor:
Dr. Mattias Svensson
Karolinska Institutet
Department of Medicine, Huddinge
Division of Center for Infectious Medicine

Co-supervisor(s):
Professor Rolf Lewensohn
Karolinska Institutet
Department of Oncology-Pathology
Karolinska University Hospital
Thoracic Oncology Unit, Tema Cancer

Professor Staffan Strömblad
Karolinska Institutet
Department of Biosciences and Nutrition

Opponent:
Professor Colin Bingle
The University of Sheffield
Department of Infection, Immunity and Cardiovascular Disease
The Medical School and The Florey Institute for Host Pathogen Interactions

Examination Board:
Dr. Anna Smed Sörensen
Karolinska Institutet
Department of Medicine, Solna
Division of Immunology and Allergy

Professor Anders Håkansson
Lunds Universitet
Department of Translational Medicine
Division of Experimental Infection Medicine

Dr. Liv Eidsmo
Karolinska Institutet
Department of Medicine, Solna
Division of Reumatology

Till Mamma

ABSTRACT

Studies of the immune system requires knowledge concerning not only the perturbating event itself, i.e. specific microorganisms, and how it interacts with immune cells but also how it functions in its natural environment – tissues. The non-hematopoietic component of tissues contributes immensely to all immune responses and acknowledging its contribution have been central to immunological research during the last decade.

The work in this thesis focuses on the use of a three-dimensional organotypic lung tissue model, which recapitulates many aspects of its in vivo correlate. **Study I** describe the properties of the organotypic tissue model and implanted monocyte-derived dendritic cells. In **Study II** we show how the organotypic tissue model can be used to study, not only secreted factors influenced by dendritic cells, but also a key functional property of dendritic cells – cell migration. In **Study III**, we used the tissue model to model a staphylococcus aureus infection, and in particular how derived toxins such as alpha-toxin and Panton-Valentine Leukocidin (PVL) contribute to tissue pathology. Immunological downstream effects of staphylococcal toxins are further explored in **Study IV**, where we investigate the role of ADAM10 and CX_3CL1 (fractalkine) in alpha-toxin mediated pathology. In **Study V**, the goal was to set up a model system in which it is possible to study the interaction between immune cells, tissue model and tumor cells, analogous to **Study III and IV**.

The studies here provide a framework for how complex, multicellular in vitro systems can be used in immunological studies in context to inflammation-driven pathologies. The validity of the model system remains to be studied, and the role for organotypic tissue models in medical research is yet to be determined. However, it is becoming increasingly clear that the study of disease mechanisms relating to inflammation will benefit from added complexity and acknowledgement that cells such as epithelial cells and fibroblast are active contributors to immune responses and tissue pathology.

LIST OF SCIENTIFIC PAPERS

Studies included in the thesis are referred to as **Study I-V**.

I. Nguyen Hoang AT, **Chen P**, Juarez J, Sachamitr P, Billing B, Bosnjak L, et al. Dendritic cell functional properties in a three-dimensional tissue model of human lung mucosa. Am J Physiol Lung Cell Mol Physiol. 2012 Jan;*302*(2):226–37. doi: 10.1152/ajplung.00059.2011.

II. Nguyen Hoang AT*****, **Chen P***, Björnfot S, Högstrand K, Lock JG, Grandien A, et al. Technical advance: live-imaging analysis of human dendritic cell migrating behavior under the influence of immune-stimulating reagents in an organotypic model of lung. J Leukoc Biol. 2014 Sep;96(3):481-9. doi: 10.1189/jlb.3TA0513-303R.
***Equal contribution**

III. Mairpady Shambat S, **Chen P**, Nguyen Hoang AT, Bergsten H, Vandenesch F, Siemens N, et al. Modelling staphylococcal pneumonia in a human 3D lung tissue model system delineates toxin-mediated pathology. Dis Model Mech. 2015 Nov;8(11):1413-25. doi: 10.1242/dmm.021923.

IV. Mairpady Shambat, S*, **Chen P***, Barilla RM, Snäll J, Welin A, Cohen TS, et al. Alpha-toxin-elicited CX3CL1-release via ADAM10 in Staphylococcus aureus pneumonia impairs bactericidal function of human monocytes. *Manuscript*
***Equal contribution**

V. **Chen P**, Svensson M. Immune-regulatory properties of human myeloid cells in a three-dimensional lung tumor model. *Manuscript*

OTHER PUBLICATIONS NOT INCLUDED IN THE THESIS

Listed in order of publication date. Studies not included in the thesis are referred to as Paper VI-XI.

VI. Kvedaraite E, Lourda M, Ideström M, **Chen P**., Olsson-Åkefeldt S, Forkel M, et al. Tissue-infiltrating neutrophils represent the main source of IL-23 in the colon of patients with IBD. Gut. 2016 Oct;65(10):1632–41.

VII. Marquardt N, Kekäläinen E, **Chen P**, Kvedaraite E, Wilson JN, Ivarsson MA, et al. Human lung natural killer cells are predominantly comprised of highly differentiated hypofunctional CD69-CD56dim cells. J Allergy Clin Immunol. 2017 Apr;139(4):1321–30.e4.

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VIII. Svensson M, **Chen P**. Human Organotypic Respiratory Models. In: Current Topics in Microbiology and Immunology. Springer, Berlin, Heidelberg 2018. p. 1-26.

IX. Svensson M, **Chen P**. Novel models to study stromal cell-leukocyte interactions in health and disease. In: Owens BMJ & Lakins MA, editors. Stromal Immunology. Advances in Experimental Medicine and Biology, vol 1060. Springer, Cham. 2018. p. 131-46.

X. Martin OCB, Bergonzini A, D'Amico F, **Chen P**, Shay JW, Dupuy J, et al. Infection with genotoxin-producing Salmonella enterica synergises with loss of the tumour suppressor APC in promoting genomic instability via the PI3K pathway in colonic epithelial cells. Cell Microbiol. 2019 Aug;21(12):e13099.

XI. Huhn O, Ivarsson MA, Gardner L, Hollinshead M, Stinchcombe JC, **Chen P**, et al. Distinctive phenotypes and functions of innate lymphoid cells in human decidua during early pregnancy. Nat Commun. 2020 Jan 20;11(1)1:381.

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10
AM	Alveolar macrophage
BAL	Bronchoalveolar lavage
CAF	Cancer-associated fibroblast
COPD	Chronic obstructive pulmonary disorder
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
ECM	Extracellular matrix
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBOC	Heriditarey breast and ovarian cancer
HIV	Human immunodeficiency virus
HMGB1	High Mobility Group Box 1
IFNg	Interferon gamma
IL	Interleukin
IM	Interstitial macrophage
IVIG	Intravenous immunoglobulin
moDC	Monocyte-derived dendritic cell
MRC-5	Medical Research Council cell strain 5
MRSA	Methicillin-resistant Staphylococcus aureus
MyD88	Myeloid differentiation primary response 88
PAMP	Pathogen-associated molecular pattern
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PMN	Polymorphonuclear cell
PRR	Pattern Recognition Receptor
PVL	Panto-Valentine leucocidin

RSV	Respiratory syncytial virus
SV40	Simian Virus 40
TAM	Tumor-associated macrophages
Th	T-helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular endothelial growth factor

1 FOREWORD

This thesis explores tissue inflammation in an experimental three-dimensional (3D) lung tissue model, especially the interplay between myeloid cells, lung epithelial cells and a defined tissue-perturbating event – such as an infection or tumor growth. Thus, critical examination of this thesis requires some basic knowledge in immunology and cell culture methods. The main goal of this *kappa* is not only to provide context to the included papers, but give you as a reader insight into my thinking regarding tissue inflammation, myeloid cells and tissue culturing as an experimental model, the central themes of this thesis. The journey began with the goal of developing (**Study I-II**) and utilizing the organotypic lung model for modelling cancer (**Study V**), but slowly emerged to be centered around tissue inflammation as work involving *Staphylococcus aureus* toxins became of particular interest in our group (**Study III-IV**). As you will learn, the work in this thesis is oriented around method development. **Study III-V** have a translational trajectory but are focused on the organotypic tissue model in two clinical settings as a proof-of-concept, rather than trying to define or delineate pathophysiological mechanisms.

The **'2. Introduction'** in this thesis serves two different purposes. i) an introduction of some basic concepts for the uninitiated in immunology and tissue biology, but with a basic background in cell biology and ii) a glimpse into my personal perspective on this subject that could explain my approach the scientific questions and aims of the different studies laid out in this thesis. For more comprehensive reviews on immunology, microbiology or tumor biology as well as cell culture techniques, I would refer to textbooks focusing on the respective subject.

In the next section, "***3 Literature Review***", I will focus on key published research pertaining to the studies included in this thesis as a backdrop for the results & discussion to each study.

'4 Material & Methods' and '5 Results & Discussion' are sections where I summarize the main points from the included papers and bring up aspects that for different reason were not included in the published version.

2 INTRODUCTION

2.1 HOST DEFENSE SYSTEMS

2.1.1 Non-hematopoietic components of the immune system

The immune system's principal function is to defend us against invading pathogens and maintaining a healthy steady state in all tissues (1). However, the immune system is heavily fortified by i) anatomical barriers, ii) biological products and iii) commensal flora and are absolutely indispensable as we would be quickly overrun by invading pathogens without any one of them. Understanding these components of the host defense system also aid in understanding how microorganisms are equipped to cause disease and increase their likelihood of survival.

Anatomical barriers refer to tissues that are exposed to the outside world, including oral mucosa, lung respiratory epithelium and the intestine. An important feature of the epithelial linings is the segregation of commensal flora and immune cells. The separation of commensal flora and the immune system allows for a non-reactive steady state, where the commensal flora can participate in the protection of tissue-harming microbes by competition of nutrients, altering of pH and hinderance of adherence (2,3). These anatomical barriers comprise of a complex and diverse set of cells, including epithelial cells, fibroblasts and endothelial cells, that continuously communicate with each other to maintain vital organ functions. The barrier primarily serves as a physical barrier to prevent pathogens reaching vulnerable vital organs, but also directly participates in the immune response by responding to the external environment and translates the information to resident immune cells. In organs such as lung and gut, the epithelium is one cell layer thick in order to allow for efficient transport of molecules between the external and internal environment. Because these organs are so exposed, they are also important avenues for pathogenic invasion and disease.

The top 2 causes of death in low-income countries are lower respiratory infections and diarrheal diseases, accounting for 9,3% and 7,2% of total deaths respectively. In the outermost branches of the respiratory tree, the host is protected by a single, ultra-thin layer of epithelial cells that allows for efficient gas-exchange between the air-filled alveolar space and the pulmonary capillaries. The space between is functionally protected by pre-formed anti-microbial products and a variety of immune cells capable of quickly eliminating pathogens with minimum induction of inflammation. In contrast to many other organs, inflammation in the lung can quickly become a hazardous host-response, as slightest fluid retention impairs gas-exchange to a significant degree. A form of hypo-responsiveness in the lung immune compartment is therefore an important concept in steady state respiratory epithelium (2,3)

The evolutionary pressure of infectious diseases in particular have likely shaped the structure of the immune system to its current form. It could be envisaged that the organization of the immune system towards combating infectious diseases have its downside in the context of non-communicable diseases, which historically likely had a lower evolutionary pressure. Diseases

such as coronary heart disease, cancer, chronic obstructive pulmonary disease (COPD) are now leading causes of death as our ability to fend of the most common infectious diseases have greatly improved. Could it be so that the way the immune system had to be organized in a certain way to cope with the constant exposure of infectious diseases and that specific organization had weaknesses that we now witness in the era of modern medicine? Thus, studying the immune system from a microorganism's perspective, will likely aid in the understanding of other inflammation-driven chronic disorders.

From an immunologist's perspective, the expansive arsenal of cell products, many of which originate from the cell types forming the physical barrier that specifically combat invading pathogens are of particular interest. These include anti-microbial enzymes, mucus and defensins that directly interacts with the infectious agent by disrupting important functions such as their cell wall integrity and ability to adhere to epithelial surfaces. Other molecules have a more indirect contribution to the first lines of defense. These molecules include cytokines, chemokines and different kind of hormones and function as first messenger to amplify more complex and specific functions that is tailored towards the infectious agent. Understanding the immunological failings and successes in an infection starts by understanding which set of molecules are released and consequently which inflammatory characteristics are induced to deal with a particular perturbating event.

Considering the plethora of microorganisms that we constantly inhale with each breath and the known colonization of nasopharynx; it was until surprisingly recently that the lung was considered a sterile environment in steady state. However, one could forgive such an assumption as the thin lining of mucus throughout the respiratory tree is constantly propelled towards the nasopharynx. This feature, combined with the frequent branching of the conducting airway make it nearly impossible for inhaled organisms, or particles of any significant size (>1μm) to reach the lower respiratory airways. The 'sterility assumption' was primarily based on that no microorganisms were successfully cultured ex vivo using conventional bacteria-culturing methods (4,5). However, the rise of culture-*independent* microbiology have completely changed this notion. Recent data suggest that bacteria found in the lower respiratory tract mirrors the microbiome found in the upper respiratory tract, but lower in density (6). Fewer studies have looked into the presence of fungus in the respiratory tract during steady state, and are more often associated with a dysfunctional immune system or co-infection (7,8). Commensal microbiota plays an important role in health and disease, but the role of the respiratory microbiota is less explored than that of the intestine and skin. For example, antibiotic treatment can result in the growth of *Clostridium difficile* or *Candida* spp in the gut – however, similar patterns are not as obvious in the respiratory tract. Clinically, antibiotic-related pneumonias does not exist and although an abnormal microbiome can be detected in several pulmonary disorders, i.e. cystic fibrosis, it is not known if changes in the commensal microbiota can contribute to the initiation or exacerbation of different pulmonary diseases.

2.1.2 The innate immune cells response

When physical barriers are breached and pre-formed cell products have been evaded – a more complex defense system is mobilized, the innate immune system. The innate immune system comprises of a group of immune cells without antigen-specificity and respond to conserved microbial structures. They have a key role in patrolling the tissue, as well as quickly respond to any sign of breach of anatomical barriers. In short, there are three groups of innate immune cells – i) granulocytes which are fast, reactive cells with high capacity to phagocytose and secrete large amounts of cytokines, ii) mononuclear phagocytic system (MPS) which have a primarily coordinating and regulatory role between the adaptive and the innate immune system and iii) innate cells of lymphoid origin such as NK-cells and innate lymphoid cells (ILCs) which are important in cell-mediated immune responses and mucosal defense.

In a generic, simplified infection scenario, the first immune cell to encounter a pathogen are dendritic cells (DCs). DCs are professional antigen-presenting cells and the archetype tissue-sampling cell, ready to receive and report signs of microbial perturbation. The so-called 'sensing' is based on the concept of Pattern Recognition Receptors (PRRs) that recognizes danger, i.e. when a cell dies and intracellular content is leaked into the extracellular space, or traces of microorganisms in the form of conserved microbial structures, for example bacterial cell wall components. The PRRs respond to Danger Associated Molecular Patterns (DAMPs) from the host and Pathogen Associated Molecular Patterns (PAMPs) from invading pathogens. Both PAMPs and DAMPs induce inflammation through various receptors. The most well-studied class of PRRs are Toll-like receptors (TLRs). Both DAMPs and PAMPs can signal via TLRs, although some DAMPs only signal through specific receptors and vice versa. Presence of DAMP-molecules can also be a sign of infection. As a general rule, activation of PRRs lead to activation of cells and induction of a proinflammatory response. The signaling pathways between infection, sterile/chronic inflammation and tissue damage are all closely intertwined, and finding parallels between autoimmune diseases, cancer and infections in terms of inflammatory profiles is not hard. For example, cancer have similarities to a viral infection in that it 'resides' intracellularly (DNA damage) and redirects the cell's protein machinery and is most efficiently combated when a strong cellular response is mounted whereas autoimmune diseases can be compared to a chronic infection where the exaggerated and prolonged inflammatory response result in collateral tissue damage.

A good example of a DAMP molecule is HMGB1, a nuclear protein that binds to the cell's DNA. During necrosis, HMGB1 is released to the extracellular space and triggers inflammation. But HMGB1 can also be released via active secretion by monocytes upon stimulation with bacterial components or inflammatory cytokines (9,10). Molecules that activate PAMP receptors are often derived from microbe-defining structures such as flagellin (TLR-5 ligand) and LPS (TLR-4 ligand). In human lung macrophages, the acute inflammatory profile is similar when stimulating with most of the TLRs (11). Similarities in the response between different TLRs could be explained by the limited set of intracellular adaptor molecules that are recruited to the TLR upon TLR engagement. Conversely, differences in responses can be attributed to the differential engagement of adaptor molecules. For example, MyD88 (one

of four TLR adaptor molecules)-dependent secretion of proinflammatory cytokines is a consequence of signaling from all TLRs except TLR3. And TLR3, TLR4, TLR7 and TLR9, but not TLR2, induces type I interferons. Furthermore, the expression pattern of Toll-like receptors (TLRs) differ between cell type and organ and could also explain differences in TLR-responses.

Although the purpose of TLR-signaling is to induce a response in order to reverse the state of the tissue into its steady state, diseases such as cancer and autoimmune diseases are associated with, albeit dysfunctional, activation of PRRs (12-14). It is important to note that immune cells are, in most cases, not the first cells to encounter and respond to microorganisms. For example, lung epithelial cells express all known Toll-like receptors (15) but their role in TLR-mediated immune activation is not clear (16,17).

Following PRR-activation, neutrophils are quickly recruited to the site, and infiltrating and/or resident mast cells and eosinophils secrete large amounts of cytokines. If this acute phase of the immune response is not sufficient, the adaptive arm of the immune system needs to be mobilized to strengthen and complement the innate immune cells. Key to the activation of the adaptive immune system is dendritic cells (DCs). DCs are commonly described as "the conductors of the immune system" but can also be likened to the "central alarm hub", that notifies the appropriate authorities to respond to ongoing perturbations. Dendritic cells are especially well equipped to be sentient units as their dendrites covers large areas and, in contrast to other sentient cells such as epithelial cells, are able to ingest, process and present the material to antigen-specific lymphocytes. This is important both for the activation of lymphocytes but also for the retention of activated lymphocytes during viral pneumonias (18).

The relationship between monocytes and dendritic cells is being increasingly nuanced the last two decades. Textbooks from the late 90s and early 00s would mention Langerhans cells as the first described dendritic cell (19), which had potent T cell-stimulatory capacity (20), in contrast to monocytes, macrophages and monocyte-derived macrophages. Monocytes were widely believed to be a precursor of certain types of macrophages and dendritic cells, shaped by the local microenvironment – and were attributed little or no specific functions other than being a precursor cell. Fast-forward 20 years and we today know, through detailed unravelling of the myeloid compartments ontogeny, that Langerhans cell are in fact more closely related to macrophages than dendritic cells (21), and that monocytes play a far more important role than just being a precursor cell (22,23). The differences between inflammation-driven differentiation of new dendritic cells and macrophages and the ontogeny of steady-state dendritic cells and macrophages is now becoming increasing clear, and much of the early work on the mononuclear phagocytic system needs to be reevaluated through this lens (24). Although monocyte-derived dendritic cells and de novo dendritic cells share many cell biological features, such as migration, phagocytosis, cytokine secretion, antigen processing and antigen presentation, their contributory role in the initiation, exacerbation or sustenance of a disease may differ significantly. During local inflammation, the number of dendritic cells increase

substantially as a consequence of monocytic differentiation (25-27). The role of circulating, conventional dendritic cells during an infection in peripheral tissue remains unclear.

2.1.3 The consequences of dysregulated adaptive immune responses.

The innate immune system's secondary function is to activate the adaptive immune system. It does so in parallel to fighting the primary infection or tissue perturbation, before it is determined if the invading pathogen overcomes the first lines of defense. The details of the different adaptive immune cells and how they function are outside the scope of this thesis, however the concept of activation and inhibition of the adaptive immune system by innate immune cells is an important feature that deserves attention.

Analogous to the parallel activation of the adaptive immune cells, systems to shut down the immune system is being set into motion before the infection is cleared. These "inhibitory" systems are required both to limit the collateral damage and to slowly shut down the potent response when the infection is over. An alternative term would be "regulatory" systems, as the main purpose is not to inhibit the response towards pathogens, but to regulate the response to make certain it is appropriate in style and adequate in strength during the different phases of the infection. As to be expected, this regulatory system can be hijacked and is a common strategy for tumor cells to perform immune evasion. Tumor immune evasion strategies are particularly interesting as an indiscriminate dampening of the tissue-inflammation is not desirable. Late-phase, chronic inflammation is associated with processes that benefits tissue repair. This include secretion of anti-apoptotic molecules, induction of angiogenesis and induction of T cell tolerance which is highly desirable features for a cancer microenvironment (28).

Initiation, induction, sustenance and termination of any immune response are shared features and are each associated with tissue-related functions other than the immune cell-specific functions that is often well studied and taught in school. Understanding the tissue-related functions gives another dimension of context when understanding the different molecules at play, and gives nuance to the differences between invading pathogens and the pathogenesis of the corresponding diseases.

Overzealous, misdirected or otherwise blunted adaptive immune responses are common features in many of the non-communicable diseases that we suffer from in the western world, including ischemic heart failure, diabetes, and neurological disorders. The origin may have dietary, infectious or degenerative reasons but in many cases ultimately lead to an unwanted adaptive immune response that initiates and exacerbates the actual pathology. What all of these conditions have in common is the chronic nature of the inflammation, and chronic inflammation always have a component of adaptive immune response to sustain the pathology. The enormous success of checkpoint inhibitors in treating malignant tumors is a testament to the potential of proper control of the adaptive immune response. Other biologicals targeting cytokines and chemokines are all strong candidates for future personalized, immunomodulatory treatment regimens, but it is becoming increasingly clear that our

understanding for how to best fine-tune and the root-cause of many diseases is very limited at best. High levels of a cytokine or relative expansion of a cell subset may merely be a consequence of a slightly unregulated, and disrupted phase of the infectious cycle and not a root cause of the disease. Understanding the immune system through the lens of microbes will very likely provide great insight into treating non-communicable diseases such as cancer, heart failure and COPD.

2.2 CELL CULTURING

All research is in some way dependent on *models*. Depending on which field of research you operate in, the model can be more or less theoretical in its nature. The overwhelming majority of models are imperfect representations of the truth, and careful interpretation of models is at the heart of any good research. In medicine, we are many times asking questions that are complex in that many systems, in which we have limited knowledge of, interact. The limited knowledge in each system can easily become amplified when studying complex human diseases. A common approach to studying immune cells are to i) isolate cells from blood ii) put them in a petri-dish like culture and iii) add a perturbating agent/molecule. Sustaining these models required well-controlled temperatures, nutritious media and reductionistic approaches that required as few "unknown" factors in order to have a reproducible experiment. However, commonly used techniques for in vitro culturing of cells can actually be considered a refinement from the first cell culture experiments that often relied on what we today refer to explant models. The monolayer single cell cultures in defined cell culturing media we are so familiar with is, to a large extent, a way of increasing reproducibility as well as specificity in our experiments.

The requirement of reproducibility is fundamental for making any conclusions from any experiments but have historically been an important limiting factor for the type of questions that can be asked. Many aspects of a cell-culturing protocol are in fact different ways to compensate for a suboptimal new environment or model. As our understanding of which factors are important for cell behavior in vivo have increased, the more ingenious ways of compensating for the suboptimal environment we come up with as biotechnology continuously revolutionize how we do research. Access to genetic engineering, and development of specific antibodies and small molecules to target desired molecules provide clarity to the answers of the questions being interrogated. It is not nostalgia, but the pursuit of better model system in which we are once again glancing on the prospect of using multicellular, complex tissues as the basis for our experiments to model immune responses, tumor development and pathogenesis of infectious diseases.

2.2.1 Conventional cell culturing

In this case, "conventional" does not necessary mean "first". Early in vitro cell cultures, dating back to late nineteenth century, included skin tissues grown in agar and coagulated serum, tumor tissue from dogs and cats in plasma from the same source and undefined cells of the neural plate of chick and frog embryos cultured using saline buffers in hanging drops. In 1907,

Ross G. Harrison pioneered the first primary in vitro cultures of developing nerve fibers. The main problem with early in vitro cell cultures were i) short life span and ii) contamination of unwanted cell types. Today, we have technologies that enables a highly reproducible environment and specificity of cell isolation. However, studying these cell types in the very advantageous environment, but not allowing for interaction between different cell types, formation of 3D structures and exposure to more complex, but physiological, environments such as air and scaffolding proteins will affect their phenotype negatively. Processes that are less dependent on its environment, such as the fundamentals of cell replication, protein synthesis and DNA repair are ideal to study in this type of experimental settings, but other more complex processes such as cell migration that heavily depend on scaffolding, cell-cell contact, spatial distribution of chemokines and their association to ECM proteins will likely be affected in most conventional cell culture-experiments. To model the complexity of any human disease is an increadible hard, if not impossible, task. The most conventional way to study diseases is to use reductionistic in vitro cell cultures of human cells to generate and test hypotheses, and validate hypotheses in animal models, which are regarded to be more complex and representative of what happens inside the human body. But animal models are expensive, laborious and may or may not be relevant in humans in the end. The pharmaceutical industry regularly tests thousands of compounds and need a relevant model system to screen for the best candidate prior starting a clinical trial, as failure in the late stages of clinical trials are extremely expensive. Specificity and sensitivity are still an issue, and better models have the potential to drastically increase the likelihood of finding good pharmaceutical candidates.

2.2.2 3D cell culturing systems and other complex model systems

As tools have developed to create more complex cell culture systems, scientist have tried to mimic different aspects of the human organs (**Paper VIII, Paper IX**). A good example of refined technology to mimic physiological environments are flow based systems. The sheer stress of blood flow has significant impact on the endothelial phenotype, and immune cell extravasation undergo a series of important changes to latch on to and interact with the endothelium to eventually penetrate through (29). Initially, flow systems were also based on the use of monolayers of endothelial cells but have not developed into complex multicellular 3D systems that recapitulates many important features of healthy blood vessels.

Multicellularity can be achieved by just mixing different types of cells and culturing them in a monolayer as conventional 2D model systems. However, the organization between different cell types in vivo are rarely stochastic and have distinct spatial organization in relation to each other. The cell-contacts in the human body are cell-cell, cell-air, cell-fluid or cell-scaffolding, but in conventional 2D models inevitable have cell-plastic contacts. The latter have been shown to negatively impact cell polarity (30), thus hindering the natural spatial organization of cells into more organ-like structures. Furthermore, it is becoming increasingly clear that 3D cell cultures have more resemblance to their corresponding clinical tissue compared to their 2D counterparts in terms of gene expression (31,32).

By mixing cells with scaffolding proteins and layer-by-layer add cells, one can artificially create spatial differentiation between cells, to then allow for natural formation of more complex and detailed tissue structures. Another common method to create 3D structures is to allow for cells to form spheroids (33,34), either by gentle vibration to hinder adherence to plastic or using the hanging-drop method. Due to their high throughput capacity and relatively complex phenotype, and the experience in toxicity screening on monolayer systems, spheroids is increasing becoming a popular alternative in drug screening (35,36).

There is little doubt that complex, multicellular 3D cell culture systems are the next generation of cell cultures. However, in the efforts towards developing new, more physiological models to study human diseases, it is important to acknowledge the limitations of these models. They are far from being 100% representation of their in vivo counterparts, and any interpretations of results from these kind of model systems should be made with caution. It may sound impossible or sci-fi to imagine that we in the future will be able to grow real tissues that are not only suitable for studying diseases but also to transplant and replace permanently damaged tissue. I am confident that the technology to execute will be there sooner rather than later, and we would do ourselves a favor to be prepared ethically for that time. The continued development and validation of methodology is both important for the interpretation of generated data, but more importantly to guide the field of organotypic model building to the next phase.

2.3 TISSUE PERTURBATION

Tissue perturbation is another way of describing all non-steady state conditions that develop over time with or without any external factors involved. The causes of cancer range from highly environmental causes in the case of asbestos-related mesothelioma to familial cancer syndromes such as Heriditary Breast and Ovarian Cancer (HBOC), but all represent a cell-perturbation event in the tissue. Infections can be caused by commensal bacteria or pathogenic bacteria representing the perturbating event, and autoimmune disorders usually need some form of outside trigger to cause disease. Of note, majority of possible tissue perturbing events will be influenced by or affect the condition of the immune system.

Significant progress in medical science, and immunology in particular, have been made by studying the behavior of immune cells isolated from blood. However, all diseases occur in, and readily involves, a specific tissue that constantly communicates with and adapts to the composition of tissue-specific functional cells and extracellular matrix. The inflammatory process is a complex phenomenon that rely on the communication and resulting action of all cells in the tissue. The host response needs to be able to deal with intracellular and extracellular bacteria and viruses, parasites, blunt trauma or sterile tissue damage, defective cells and potentially cancerous cells, all whilst still performing important homeostatic functions when not currently being threatened. The multifaceted perturbations are all met with variations of immune responses that often overlaps with each other. Understanding the phenotype of different inflammatory responses will aid us tremendously in the future to develop the next generation personalized medicine.

2.3.1 Invading microorganisms

Non-sterile inflammation primarily deals with infections with bacterias, viruses, parasites and fungi. These microorganisms all have their unique components that can be recognized by the immune system and elicit different types of immune responses. For combating viruses, antigen processing, reduced translational activity and cellular immune responses are particularly important, whereas most bacteria, parasites and fungi require a more humoral response with induction of enhanced phagocytic activity. Fungi and parasite infections in lung are often associated with a compromised immune system and/or co-infection with bacteria. In developing countries, lower respiratory tract infections cause more death than malaria, HIV and tuberculosis combined and are primarily caused by streptococcus pneumoniae, haemophilus influenzae type b and RSV. Staphylococcal pneumonias are historically relative infrequent, but act as a severe complication of influenza (37), ventilator-induced pneumonia (38) and hospital-acquired pneumonia (39). In contrast to streptococcal pneumonias, staphylococcal pneumonias often have a more severe clinical presentation and is an important cause of death worldwide.

2.3.2 Tumor growth

Tumors represent a different type of tissue perturbation. It causes disease by causing a loss of function in the primary and secondary tumor-bearing organs and can lead to significant changes in blood chemistry. Cell growth and morphogenesis is characterized by a controlled replication of cells, that respects the tissue boundaries and organization. Tumor growth do not respect its surroundings and causes tissue damage partially by sheer uncontrolled growth. Furthermore, drugs often have a hard time penetrating the tumor mass, as the vascularization is unorganized and immature. It is extremely important to find cancer in an early stage, when conservative surgary is enough to cure the patient. However, many cancer forms are quiet and do not cause any noticeable symptoms, and thus only detected on radiological examination or at a much later stage when clinical symtoms become obvious.

Each time a cell divide, there is a small chance of errors being built into the DNA. Ideally, those cells should preferable undergo apoptosis and be replaced by a normal cell. When they remain as a dysplastic cell, it is often removed by immune cells that can detect non-self peptides or stressed cells by examining the cell surface. In fact, immune cells constantly remove defective and potentially cancerous cells, which bears the question: what is the role of the immune system when a cancer have managed to escape the safety mechanisms built in? Key hallmarks of all cancers are immune evasion and tumorigenic inflammation. The tumorigenic inflammation can itself promote immune evasion but certain types of inflammation have tissue-regenerative potential that promotes cell division, angiogenesis and immune dampening properties (28,41). Modulating the immune dampening properties, and/or using checkpoint inhibitors to lift the immune evasion have proven to be a successful avenue for a new generation of cancer treatments. Especially the use of CTLA-4 inhibitors and PD-1/PD-L1 inhibitors have proven promising and useful in a diverse set of cancers, despite immune-related adverse effects (42). This suggests that dampening of the immune system to play an important role in cancer

progression. The usefulness of these checkpoint inhibitors is far from universal. It is either so that the immune evasion strategies are diverse and complex or that some cancer forms depend less on its immune dampening properties (43). These are difficult questions to answer but identifying which cancer forms are most likely to respond to immune modulating drugs remains a priority in cancer research.

Studying cancer is an extremely difficult task, and the reasons are well summarized in the seminal review articles by Hanahan and Weinberg (44,45). A reason that touches upon many of the proposed hallmarks is the heterogeneity within the cancer. Cancer is more similar to a complex organ, than that of a clonal expansion of a dysplastic cell. Cancer cells themselves are heterogeneous, but there are also important co-conspirators within the cancer tissue, such as cancer-associated fibroblasts (CAF) (46) and tumor-associated macrophages (TAMs) (47,48). The multicellularity and heterogeneity between cancers contribute to the difficulty in creating representative model systems to study cancer progression and its interaction with the immune system.

3 LITTERATURE REVIEW

3.1 THE RELEVANCE OF ORGANOTYPIC TISSUE MODELS IN STUDYING IMMUNE CELL BEHAVIOR IN HEALTH AND DISEASE

All the studies conducted in this thesis revolves around the utilization of an organotypic tissue model presented in **Study I**, which in turn was a continued development of the work by Choe *et al* (49). The development and utilization of more advanced in vitro models are well in line with the concept of the 3 R's in animal research (Replace, Reduce and Refine) which aims to provide a framework for more humane and ethical animal research. In the context of the organotypic model system we have developed, one should note that the purpose is not to replace animal models but to complement and provide alternative to expensive and not always ideal disease models. Importantly, host tropism and species-unique signature of the immune system may complicate the interpretation of animal models.

Organotypic tissue model systems bridge the reductionistic 2D in vitro cultures and animal models. What organotypic tissue models lack in complexity compared to animal models, they gain in species relevance and experimental setup flexibility. And what they gain in complexity compared to 2D in vitro cultures, they lack in scalability. There is no single system that is overall superior compared to another.

As described in **Study III**, the pathogenesis of staphylococcal pneumonia is highly dependent on secreted exotoxins among which, PVL do not cause lysis of neutrophils in murine and simian models, but does so in humans and rabbits (50). Thus, studying the totality of S. aureus toxicity in mice may result in an overestimation of the contribution of the other toxins released by S. aureus, such as alpha-toxin.

Since the publication of our organotypic lung tissue model, most studies using a similar setup primarily focuses on toxicology-related questions in COPD and cigarette smoking. Studies using 'immunocompetent' models remains few, but attempt have been made to study mycobacterial infections (51) and aspergillus fumigatus infections (52).

The usefulness of immune competent tissue models to study immune cell behavior remains to be determined. Promisingly, the model setup has been successfully replicated in different labs around the world, and an effort towards validating gene expression profiles of implanted immune cells and stromal cells with its corresponding in vivo cells in both steady state and in pathological conditions should be prioritized to incentivize more research using these kind of model systems.

3.2 ROLE OF CHEMOKINES IN SHAPING THE INNATE IMMUNE SYSTEM

Chemokines are chemotactic cytokines that directs and positions immune cells in different locales. During acute inflammation in the lung, mast cells are well positioned to quickly respond without any transcriptional activity by releasing its pre-formed content. In mouse models of airway hyperreactivity or LPS stimulation, CCL1, CXCL1 and CXCL2 have been

shown to be released by mast cells to promote early neutrophils recruitment (53,54). For sustained influx of innate immune cells, engagement of local immune cells other than mast cells via PRRs is crucial. Acute inflammatory cytokines such as IL-1 and TNF are released by activated dendritic cells and macrophages which stimulate other resident immune cells and/or activates the epithelium and stromal cells. Cytokine-activated epithelium then produces a plethora of chemokines, including CXCL8, CXCL1, CXCL2, CXCL3 and CCL5 (55-57). Beside neutrophils, monocytes are recruited early to the inflamed tissue but require a different set of chemokines. Two important chemokines with known monocyte migration-inducing capacity are CCL2 and CX_3CL1/fractalkine. When CCL2 is produced, it dimerizes and bind to the extracellular matrix and form a chemokine gradient to guide the migration of monocytes (58). All monocyte subsets express the fractalkine receptor CX_3CR1 which is believed to convey survival signals for patrolling monocytes (also known as intermediary monocytes) (59) and early migration of classical monocytes (60). Functions of chemokines beyond cell migration is a concept we explore further in the context of staphylococcal pneumonia in **Study IV**. For example, monocytes do not migrate towards CXCL8 gradients, despite expressing CXCR2. However, CXCL8/CXCR2 interaction have been shown to be an important interaction during the adhesion of monocytes to the vascular endothelium (61). Fractalkine have similarly been described as an anchoring protein, but its unique regulation as a membrane-bound and soluble chemokine suggest multiple mechanisms in which fractalkine can shape the monocytic response in inflammatory conditions (62,63).

In vitro studies of chemokines and immune cell activation is particularly well suited in tissue models comprising of scaffolding proteins to support the network of fibroblasts and epithelial cells. A common method to study cell migration is boyden chamber (transwell) migration assays. The principle is simple; by separating cells to an upper reservoir with a porous membrane with a chemokine-rich supernatant in the lower reservoir, migrating cells are expected to move from the upper reservoir to the lower reservoir. The chemokine gradient is supposedly being formed by diffusion in the supernatant. However, this type of gradient is not stable and not comparable to the gradient formed with the help of extracellular matrix proteins. It is therefore difficult to assess if cells are plainly activated and start to move in randomly, or if they in fact migrate towards the source of stimuli. Furthermore, proteolysis is an important aspect of monocytic cell migration, in which podosomes play an important role. Podosomes in 2D cultures have primarily been defined as adhesion- and mechanosensing molecules and traction-generating structures. In 3D matrices, the podosomes are arranged slightly different and are larger in size compared to its 2D counterpart. In 3D the podosomes are focused around the tip of the cell protrusions that are largely absent from 2D cultured cells (64,65). Studying cell migration in physiological relevant context could therefore be especially important in order to recapitulate in vivo migration.

As the concentration of released chemokines are naturally higher around the source, it can be misleading to measure chemokine levels in supernatants to determine or create chemotactic activity. Allowing for a local source of chemokines, like cytokine or PRR-activated epithelium, a more realistic chemotactic scenario can be modelled. With the aim of studying a functional

response of implanted dendritic cells, we sought to investigate the possibility to study migratory responses following external stimuli of the 3D organotypic tissue model in **Study II**.

3.3 PATHOGENESIS OF STAPHYLOCOCCUS AUREUS

Despite being one of the most common and important human pathogens, it is not entirely clear why some are merely carriers are *S. aureus* and some acquire an invasive, potentially life-threatening infection. A known complicating factor is co-infection with influenza viruses. During the Spanish flue in 1918, an estimated 50 million or 95% of infected people died from a pneumonia exacerbated by bacterial co-infection(66). At the time of writing this thesis, we are in the middle of a pandemic caused by the 2019 novel coronavirus, SARS-CoV-2. During the 2002 outbreak of severe acute respiratory syndrome (SARS) in China, a hospital in Hong Kong reported that MRSA was found in 25% of hospitalized ICU-patients during the SARS period, and 3,5% and 2,2% pre- and post-SARS period respectively (67). These numbers are consistent with the expected magnitude of bacterial superinfection during influenzae infections (68), and are to be expected during the current pandemic. As the pneumococcal vaccine is gaining increased penetrance in the population, Staphylococcus aureus have risen to prominence as a common and deadly cause of secondary bacterial pneumonia. One reason for this is likely due to the emergence of USA300 clonotype, causing community-acquired methicillin-resistant Staphylococcus aureus (CA-MRSA) infection. It was noted early that USA300 express high levels of Panton-Valentine leukotoxin (PVL), a known S. aureus toxin, which was and is still believed to be an important cause of necrotic lesions associated with the disease (69). However, the precise mechanisms and relative contribution of PVL in relation to other S. aureus toxins during necrotizing pneumonia is fiercely debated. Studies on PVL are complicated by host specificity, as its receptor C5aR is heterogeneously expressed among different species. Studies using humanized mice, i. e mice with a reconstituted human immune system, show increased susceptibility to PVL-mediated pathology (70). Thus, its precise role in human pathology remains to be fully understood, and relevant model systems will significantly aid that endeavour. Bacterial co-infections during viral pneumonias may also have deleterious effects as the host immune system is adapting towards a viral infection and produces responses that is not well suited to simultaneously combat bacterial infections. Influenza virus infection causes damage of the anatomical barrier and dead cells mixed into the fibrinous mucus provide a nutrient-rich environment for bacteria to thrive (71). It is hard to attribute strain-specific bacterial phenotypes to the pathogenesis of bacterial co-infections but is likely to play as important role in determining the outcome as virus-specific attributes.

Another important S. aureus toxin associated with USA300 is alpha-toxin (72,73). Like PVL, alpha-toxin is a pore-forming toxin and causes cell lysis. However, unlike PVL, alpha-toxin binds to ADAM10 which expressed on most tissues and is a mechanism of action we explore further in **Study IV**. The role of ADAM10-mediated tissue-pathology is especially evident when the toxin-concentration is low as ADAM10 functions as a high-affinity receptor. It is further complicated by the initiation of intracellular signalling cascades that results in the

disruption of focal adhesions (74). Although toxins are only a part of the story in terms of *S. aureus* pathogenesis, it is important to characterize the downstream effects of *S. aureus* toxins in detail in order to find new therapeutic targets.

3.4 IMMUNE-REGULATING PROPERTIES OF THE TUMOR MICROENVIRONMENT.

The immune system possesses an enormous capacity to recognize and respond to non-self-antigens. Tumor neoantigens, or tumor-specific antigens, are peptides that are not expressed by normal tissue or organs and represent the best avenue for inducing lasting immunity against tumors following successful treatments. Early studies showed that mice were protected from re-exposure of tumors that have previously been surgically removed or if first exposure of tumors were lethally irradiated, suggesting that a memory response could protect the host against cancer (75). These scenarios are of course extremely artificial and not applicable to normal disease progression. Additionally, an important tumor-promoting hallmark is immune suppression. Even if the nature of tumor neoantigens is still very unclear, the dysplastic nature of cancer cells combined with the presence of foreign antigens should have the potential to induce strong immune responses, as long as the neoantigens are made available and immunogenic and that whatever immunosuppressive mechanisms in play can be thwarted. Recent successes in the use of checkpoint inhibitors corroborates this hypothesis as it has been shown that tumor mutational burden is correlated to response with anti-CTLA-4 and anti-PD-1 therapy (76,77). This suggests that the immune system can, but do not, kill cancer cells, and that immune suppression play an important role in immune evasion in cancer.

It is well acknowledged that tumor tissue has organ-like level of organization and complexity. Cancer treatment is reaching a paradigm shift, and focus have been on non-cancerous cells, such as epithelial cells, immune cells and fibroblasts. By targeting the co-conspirators, a whole new treatment arm is slowly being developed for fighting cancer.

In the tumor microenvironment, lymphocytes express high levels of inhibitory receptors and cannot perform their normal function. However, it is not well known what contributes to the immune suppressive phenotype of these lymphocytes. Myeloid cells have been implicated as a potential suppressive contributor in cancer inflammation. The term “myeloid-derived suppressor cells” is as widely used as it is difficult to define accurately. What is clear, is that innate immune cells have mechanisms of inhibiting the adaptive arm of the immune system for proper resolution of inflammation, as well as protection against overzealous or unregulated adaptive responses.

4 MATERIAL & METHODS

4.1 ORGANOTYPIC TISSUE MODEL

Study I describe the characterization of a "immune competent" organotypic lung tissue model, aiming to recapitulate airway wall and mucosa. The foundational structure of the lung tissue model was based on the work by Choe *et al* (49) and includes the addition of monocyte-derived dendritic cells. The model setup by Choe *et al* required the sequential addition of acellular and cellular layers of collagen I, 16HBE cells, and air-exposure. Proper maintenance of the lung tissue model enables experiments to be carried out well over 4 weeks after maturation. As our goal was to incorporate dendritic cells into the model, and there was no continuous supply of immune cells to the lung model, we opted to do all analysis within 6-11 days post implantation of the dendritic cells.

The model was developed to provide a tool for studying immune responses in a more physiological and relevant context compared to conventional monolayer cell culturing methods. Moreover, it adds a new dimension of immune system-pathogen interaction; non-hematopoietic host cells. Together, it forms the basis of host-pathogen interaction. There are three main components of the non-hematopoietic compartment in the lung tissue model used in this thesis; lung epithelial cells (16HBE), fibroblasts (MRC-5) and scaffolding (collagen I). The model aims to represent the larger bronchial airways or conducting airways where no substantial gas-exchange occurs. Lung epithelial cells used in the organotypic tissue model are a simian virus 40 (SV40)-immortalized human bronchial epithelial cell. Before the discovery of SV40 in 1960, millions of people were administered SV40-contaminated polio vaccines which caused a significant public health concern as SV40 was shown to cause cancer in laboratory animals. Engels *et al* have published a series of epidemiological studies indicating that SV40 likely does not cause human cancers (78), however contrasted by Meneses *et al* who showed SV40 DNA sequences to be significantly more often detected in lymphomas than in control samples (79). In contrast to other cell lines used to represent respiratory epithelium, 16HBE is derived from a healthy cell, but its histological profile in the organotypic tissue model suggest that there are significant differences compared to the bronchial epithelium found in vivo. The two most obvious differences are the stratification of 16HBE cells in the model, and the lack of ciliation. Respiratory epithelium should be pseudostratified, meaning that all cells are resting on the basal membrane. In the organotypic lung tissue model, 16HBE cells expand and stratify upon air-exposure, and if left for long enough exposed to air (>7 days), the epithelium become 5-10 layers thick. An important feature of pseudostratified epithelium is the ability to absorb and secrete biological products to maintain tissue homeostasis. Another aspect of respiratory epithelium is that it is not homogenous but contain different cell types with various functions. For example, goblet cells are specialized in producing mucus and basal cells are precursor cells embedded in the pseudostratified epithelium. Acknowledging these differences significantly impacts which type of questions that can be asked using the organotypic tissue model, and how the experimental outcomes are limited when testing other hypotheses.

Studies on microbial colonization is challenging, as the environment is still very beneficial for most microbes and requires extensive optimization and limitation on type of assays for the model to not be overgrown with bacteria. To study how stromal cells and immune cells interact, we have used two principally different strategies in the included studies. One way was to allow for implantation of monocytes or dendritic cells into the organotypic lung tissue model and measure the effects following isolation of the cells or compared to a control tissue. This approach was particularly important in **Study II and V**, as we expect cell migration to be one of the cell functions that will differ significantly when comparing conventional in vitro experiments and the in vivo setting. The second approach was to take supernatant from the organotypic tissue model exposed to a perturbance and "expose" freshly isolated monocytes to the tissue-derived soluble factors. Although the lack of cell-contact dependent mechanisms that may influence the response, we have thought of it as a model where blood monocytes arrive to a specific tissue and are exposed to all the factors induced by for example a staphylococcal toxin. The second approach was significantly easier to scale up, and where cell number never is an issue. The first approach is potentially the most desirable option, but depending a labs technical limitation, it is not always the best option.

Respiratory epithelium also has a temperature slightly below core temperature, as cold or room-temperature air is constantly flowing across the epithelium. Although the temperature difference is not as big as compared to skin, a couple of degrees may still influence immune cell function (80,81). The lack of blood flow is addressed by regularly changing the media that is in contact with the basolateral side of the model, but compared to normal blood vessels, it does not provide an avenue for replenishment of immune cells that would normally contribute to the removal of dead cells. Thus, cell debris will accumulate in the model and likely contribute to the steady state profile that we observed.

4.2 MONONUCLEAR PHAGOCYTIC CELLS

Magnetic cell separation, also called immunomagnetic cell separation, provide one of the most common and reliable methods for isolating specific cell population with high purity. As we sought to recapitulate a steady state tissue that could respond to exogenous substances and pathogens, we tried to populate the tissue with dendritic cells by differentiation monocytes isolated from blood.

A reason for not choosing to model tissue-resident adaptive immune cells is the mis-match compatibility issue with tissue cells from another host. Granulocytes proved to be short lived and easily activated. Other immune cell candidates included monocytes and macrophages. Monocytes were and is still considered to not have a lung-resident specific phenotype, and more prevalent during inflammation rather than steady state. The use of macrophages was complicated by the differentiation protocols that forced the cells into extremely polarized phenotypes which would be difficult to assess after exposure to exogenous stimuli. Furthermore, at the time of developing the lung tissue model, little was known about interstitial macrophages, their ontogeny, localization and phenotype. Most macrophage-related work in lung have been directed towards alveolar macrophages (AMs), in part due to the high numbers

of AMs attained during a BAL wash. Only recently have interstitial macrophages been more thoroughly characterized (82). In the end, our longstanding interest in cell chemotaxis and the immune surveillance capabilities made us consider DCs as our initial point of focus.

Dendritic cells as a whole are extremely rare, and the amount of lung tissue needed in order to isolate enough primary tissue-resident dendritic cells would be impractical. Even the frequency of blood DCs are too low for reliably producing any larger numbers of tissue models. Monocyte-derived dendritic cells have long been used as a substitute for de novo DCs and there are well established protocols to reliably attain high numbers of moDCs. As these cells were differentiated with IL-4 and GM-CSF, at best they represent a DC-like cell under inflammatory conditions. For the purpose of characterizing immune implantation in the model, and studies of DC migration, the limited relevancy of moDCs was acceptable to us. For studies where our main interest lies in finding an altered phenotype as a consequence of a tissue response, we opted to use freshly isolated monocytes that where stimulated with supernatants from the organotypic tissue models. Instead of creating a model to study resident immune cells, this approach would represent infiltrating monocytes to the lung tissue that are exposed to the soluble factors produced by epithelial cells and fibroblasts. Experimentally, it proved to be a highly reproducible system that allowed for multiparametric flow cytometry analysis and functional assays of the monocytes after exposure to the tissue microenvironment.

5 RESULTS & DISCUSSION

5.1 STUDY I

This study describes a diverse array of methods to study functional properties of dendritic cells in a physiological relevant milieu. By histologically characterizing the organotypic lung tissue model, we conclude that it shares many features with real lung tissue and that its three-dimensional organization is self-sustainable and show characteristics not present in reductionistic monolayer systems. For example, the formation of a basal membrane on the basolateral side of the 16HBE epithelial layer and a mucous layer on the apical side are not observed in conventional cell culture settings. The main structural component of the tissue model when forming it is collagen I, but over time as the tissue model matures – extracellular matrix proteins such as collagen IV, vimentin and tropoelastin is deposited and provide a much more complex and physiological environment than conventional cell culture methods can do. Immune cells invariably interact with ECM proteins, and it provide a particularly important function in the context of chemokine regulation.

The organotypic lung model consist of three different cell types; epithelial cells, fibroblasts and monocyte-derived dendritic cells (moDCs). Importantly, the moDCs survive without the addition of exogenous growth factors (Figure 4D). Cryosectioning and whole-mount live imaging show that dendritic cells are closely associated to the epithelium throughout the maturation of the model. If the maintenance of dendritic cells in the lung model is mediated via cell-cell contacts or soluble factors was not tested, but the high expression of GM-CSF mRNA in the organotypic lung model suggest that survival-promoting signals are present in the lung model.

Although occasional biopsies from the bronchial tree can have a stratified epithelial appearance, bronchial epithelium is a pseudostratified epithelium. Prolonged air-exposure of the organotypic lung model tend to induce a stratified squamous epithelium-looking appearance and reaches a maximum of 10 layers. The 16HBE cells used to form the epithelial layer does not ciliate in our model, which is also a sign of an undifferentiated bronchial epithelial cells. Thus, the epithelial layer in the model effectively function as a physical barrier, but its representation of the in vivo bronchial epithelium and its barrier function can be questioned, even if the organotypic model as a whole supports the survival of dendritic cells.

The pathological interplay between dendritic cells and epithelial cells is a well-known phenomena in asthmatic inflammatory reactions (83-87). Although much focuses on the secreted factors by epithelial cells that induces dendritic cell activation, the communication goes both ways. Less is known about the interaction between dendritic cells and epithelial cells in steady state. In Figure 7, we demonstrate how the chemokines secreted from the organotypic model is affected by the presence of dendritic cells. The number of dendritic cells decrease over time (Figure 5) which would suggest that any increases in the chemokine secretion is more likely to be secreted by epithelial cells. However, CCL18 is a chemokine predominantly secreted by monocytes/macrophages and DCs. The organotypic model alone

expectedly did not produce any CCL18, and moDCs alone showed low but detectable levels. But when the dendritic cells were implanted to the organotypic model, the expression increased drastically. It is unlikely that detected CCL18 come from the dendritic cells before they were implanted, as the number of dendritic cells decreases over time and media is changed every 48 hours post implantation. Thus, the remaining dendritic cells are likely modulated by the tissue model to secrete more CCL18. In addition, supernatants from separately cultured cells (16HBE and MRC-5) were not able to induce CCL18 expression in DCs, whereas supernatant from organotypic model with 16HBE + MRC-5 cells could, another example of the phenotypic difference cells have in 3D matrices compared to conventional 2D in vitro cultures.

In contrast to CCL18, which is found at high levels in plasma during steady state, CCL17 and CCL22 are induced upon inflammation, mainly in relation to Th2-activation. These chemokines were not further induced by the organotypic tissue model as CCL18 was. It is tempting to suggest that the organotypic model induces a steady-state phenotype in the dendritic cells. In some aspects, and in relation to the high inflammatory phenotype of moDCs, that could be the case, but the high expression of IL-1ß, TNF and CXCL8 mRNA in the model contradicts that notion. A constant low-grade inflammatory environment is possible, although the immunological and tissue-response needs to be tightly regulated in the lung and consequently balanced with other immunosuppressive actions (88,89).

The relevancy of GM-CSF and IL-4 differentiated dendritic cells can also be questioned. These cells most likely have an inflammatory phenotype compared to de novo dendritic cells and have previously been expected to only appear during infection or sterile inflammation (66-68). However, monocytes have been shown to differentiate to dendritic cells under steady state conditions in lung (25). Whether this represent the “non-sterile” environment of lung, or if there are specific properties in the steady-state lung microenvironment, remains to be investigated. Tissue-resident immune cells in the lung microenvironment are of particular interest in autoimmune reactions, such as asthma, and their phenotype is becoming increasingly well understood (90,91), but the inaccessibility of human tissue-resident cells make them difficult to study mechanistically. Although the resemblance of the organotypic lung model and real lung tissue have clear limits, the findings in Study I warrants further exploration of the phenotypic changes induced in the dendritic cells following implantation to a 3D multicellular and physiologically relevant milieu.

5.2 STUDY II

Study II show that dendritic cells implanted in the organotypic lung model are functional, and that its migratory properties can be visualized and quantified whilst still in the organotypic model.

In study I, we could measure the impact of the 3D organotypic model on dendritic cells in terms of survival and chemokine expression. **Study I** suggest that DCs in the organotypic model are functional, although the decline of DCs over time could potentially indicate that the cells are dying and/or non-functional. An archetype function of DCs is migration and considering the

expression of chemokines in the organotypic lung model, we sought to find direct evidence of functional DCs in the model by quantifying DC migration using live confocal microscopy. By utilizing genetically modifying 16HBE and MRC-5 cells to express GFP and orange fluorescent proteins, respectively, we could observe two distinctly separated cell layers – a fibroblast-layer and an epithelial layer. The confocal microscope was able to penetrate approximately 150µl beneath the top part of the epithelial layer. As dendritic cells were constantly closely associated to the epithelial layer (≈20-40 µm thick), all dendritic cells could easily be visualized.

Tissue-sectioning using cryopreserved or paraffin-embedded tissues have severe limitations when examining rare cell subsets. The way the model is built makes it particularly difficult to study the cells via immunohistochemistry, as each section consist only of a minimal area where DCs could possibly be detected. For quantification purposes, immunohistochemistry of fixed, sectioned tissue is not suitable. Relative numbers of DCs could be quantified using multiparametric flow cytometry, but the loss of cells during tissue processing makes flow cytometry less than ideal for absolute counts of rare cell subsets. Therefore, in **Study I**, we developed a method to visualize all implanted dendritic cells using cell tracker dyes and GFP-expressing 16HBE cells. By transferring the model and some media onto a microscope-friendly 6-well plates, multiple models can be imaged simultaneously and the DCs relative position to

Even in the absence of any stimulating agents, we could see that DCs were spontaneously moving around in the model over time. When stimulating with TLR2 and TLR4 ligands, to provoke an epithelial response and DC activation, we observed that DCs were closer, and more often inside, the epithelium compared to the unstimulated models. 16HBE cells express both TLR2 and TLR4, and are able to respond to for example LPS. However, they also express lower levels of MD-2, an adaptor molecule for TLR4. It has been suggested that the general hypo-responsiveness in lung is an important homeostatic mechanism and partly mediated by the differential TLR-activation in lung epithelial cells compared to other organs (15,16,92,93). The association of DCs to the epithelial could be either an indirect effect of epithelial-induced chemokine secretion, or a general increase in motility of DCs through direct TLR-activation and that the presence of the anchoring chemokine fractalkine (CX_3CL1) (**Study I**: Figure 4D, **Study IV**: Figure 2A) leads to a higher ratio of DCs associated with the epithelium. It should also be noted that the relative position of every single DC varies widely within each model, irrespective of stimuli. This could represent potential DC heterogeneity, cell viability or stiffness of the model. In contrast to 2D migration, DCs in the organotypic model are forced to interact with extracellular matrix in all directions and may even require some degree of proteolysis for any significant cell displacement. Although meaningful directionality can only be quantified in the Z-axis (apical-basolateral), the addition of XY-plane displacement enables the quantification of the total distance travelled and speed of each individual DC. DCs from TLR-stimulated models exhibited both faster and longer distances travelled per time unit. Additionally, migrating cells constantly reorganizes the cytoskeleton and larger protrusions can be observed in migrating cells compared to stationary cells. The cell-tracker dyes enabled us to create 3D surfaces of the individual cells, representing the outline of each cell. Activated

cells, and motile cells, were less spherical which correlated with the mean speed of the individual DC in all stimulated models (Fig 4).

Using the 3D organotypic model setup combined with confocal microscopy, spatial and temporal quantification of DC migration under tissue perturbation is possible. The main drawback with cell-tracker dye systems is that is indiscriminately stain all living cells. Even if cell purity is high, it cannot account for phenotypic changes and cell viability. For example, activation of DCs with TLR-ligands induces upregulation of co-stimulatory molecules CD80, CD86 as well as upregulation of HLA-DR and CCR7. The activated phenotype should theoretically be linked to the increased migratory capacity. Although we could detect an activated phenotype of DCs after isolation from the model and staining for flow cytometry (Figure 5), it is no longer possible to dissect the migratory properties in relation to their phenotype on a single-cell basis.

5.3 STUDY III

Our increasing experience with the organotypic tissue model led us to test the usefulness of the model in a pathogenic setting. One of the most serious respiratory infections are staphylococcal pneumonias, but the pathogenesis behind severe necrotizing pneumonia remains controversial. This is partly due to lack of relevant model systems, but also due to the abundance of toxins expressed by S. aureus. Differences in clinical presentation, severity and relative expression of virulence factors further complicates the dissection of staphylococcal pneumonia pathogenesis.

As addition of live, whole bacteria to the model would quickly render the model system overpopulated with bacteria and unusable for further analysis, we used a bacterial supernatant to stimulate the organotypic lung model. By culturing 31 different S. aureus isolates with different clinical presentations and toxin-properties, we found that supernatants with high levels of bacterial toxins correlated with tissue disintegration. The damage seen in the organotypic tissue model could be correlated with the clinical presentation of the patients from which the S. aureus strains were isolated from. A frequent debate in S. aureus pneumonia pathology is the relative contribution of alpha-toxin vs PVL as a cause for the severe clinical outcome. Our data suggest that alpha-toxin causes tissue damage through its direct interaction with epithelial cells, whereas PVL causes indirect epithelial injury as supernatant from PVL-treated neutrophils, but not PVL alone or control-treated neutrophils, were as effective as alpha-toxin in inducing epithelial injury. Thus, suggesting that two toxins independently of each other contributes to tissue pathology and exacerbates one another. Using the same experimental setup as in Study II, but without the addition of dendritic cells, we could quantify the tissue damage over time as a function of loss of GFP in the epithelial cells. Live imaging and immunohistochemistry showed reduced signs of tissue disruption and loss of E-cadherin in S. aureus strains expressing high levels of alpha toxins and stimulation with pure alpha-toxin, but not with recombinant PVL or supernatants from S. aureus strains expressing high levels of PVL, and low levels of alpha-toxin. Notably, the effect on cell-cell interacting molecules were not global as evident by the relatively unchanged claudin 1 staining-pattern after stimulation with bacterial supernatant. Pre-treatment of the bacterial supernatant with IVIG blunts alpha-

toxin mediated cytotoxicity in epithelial cells and PVL-mediated cytotoxicity in neutrophils. Neutrophil recruitment is essential in all types of bacterial infections, but their sensitivity towards PVL may cause more collateral damage than intended. The solution is not to hinder the recruitment of neutrophils, but to find ways of protect neutrophils whilst they are combating the bacteria. Since this published work, IVIG have been shown to confer protection against necrotizing phenotype of MRSA pneumonia in a rabbit model (94). Nosocomial S. aureus infection in neonates have also been shown to benefit from IVIG (95). Although any findings made in the organotypic tissue model pertaining to mechanisms of pathogenesis needs to be tested and validated in other model systems and in vivo, the organotypic model can cleary be used as a hypothesis-generating platform for deciphering complex pathogenic mechanisms of S. aureus. One example of such hypothesis-generating experiments could be to identify which molecules present in the PVL-stimulated PMN supernatant are most important for exacerbating toxin-mediated tissue injury.

5.4 STUDY IV

The stimulation of the lung tissue model with bacterial toxins caused severe tissue damage by evidence of histological examination. The loss of E-cadherin and relatively unaffected levels of claudin 1 suggest that there are enzyme-specific or cell-modulatory events in play. ADAM10 have previously been identified as a receptor for alpha-toxin, and it coincidentally also targets E-cadherin when activated. To test the hypothesis if ADAM10 confers the alpha-toxin mediated tissue injury, we set up a series of experiments aimed at blocking the down-stream pathway of ADAM10. Indeed, inhibition of ADAM10 protected the tissue model from injury. As ADAM10 have multiple substrates, and alpha-toxin specifically binds to ADAM10, we speculated that downstream of ADAM10 activation may have implications related to the immunity of S. aureus infection. In Study I, we had already confirmed the expression of fractalkine on mRNA level. Fractalkine is a known target of ADAM10, which naturally made us ask the question what potential role of ADAM10-mediated fractalkine cleavage had in S. aureus infection. Notably, plasma levels of fractalkine is elevated in patients with S. aureus infection compared to healthy controls. Pachot *et al* have seen a similar pattern in a sepsis cohort, and speculate that the loss of membrane-bound fractalkine from the endothelium may contribute to the inability to kill invading microorganisms as fractalkine have been suggested to induce arrest and transmigration of patrolling monocytes. However, fractalkine is unique in that it is both functional in its membrane-bound form and soluble form. We show that monocytes can migrate towards a CX_3CL1 gradient, and that blockade of ADAM10 activity or alpha-toxin activity shunts CX_3CL1-mediated migration. Fractalkine have also been shown to have non-chemotactic functions in NK cells, as it induces IFNg production in its membrane/immobilized form compared to the soluble variant. As fractalkine is released in systemic S. aureus infections and S. aureus pneumonias, we stimulated monocytes with fractalkine and observed phenotypic and functional changes that could be relevant for their anti-bacterial function. Of particular note, we found that monocytes that were pre-treated with fractalkine and then infected with S. aureus had lower production of NO and ROS. Taken

together, the findings in **Study IV** proposes fractalkine cleavage to contribute to S. aureus pathogenesis by modulating monocyte function.

This study is very much focused on the single downstream events of alpha-toxin and ADAM10 leading to fractalkine cleavage. As bacterial toxins in S. aureus are numerous and the substrate specificity of ADAM10 is broad, we do not exclude that the fractalkine effect in vivo is negligible or redundant. However, the notion that chemokines have functions beyond cell migration is worth exploring and could drastically improve our understanding of complex networks of chemokine profiles in health and disease.

5.5 STUDY V

Here we describe a model system for studying tumor growth and interaction with immune cells. By using a similar setup as in **Study II**, we implanted different tumor spheroids formed using the hanging drop-technique. This enabled us to visualize defined foci of tumor-rich areas using confocal microscopy and quantify how the dendritic cells spatially organized themselves in relation to the tumor spheroid. Dendritic cells in models that were implanted with A549 tumor spheroids, were found at a higher frequency in the tumor area compared to H1299 tumor spheroids and 16HBE control spheroids. We could also observe changes in soluble factors secreted by the model depending on the implanted tumor spheroids in terms of chemokines and ability to alter monocyte phenotype. The H1299 tumor model supernatant showed higher levels of secreted chemokines and had higher impact on expression of co-stimulatory molecules on monocytes following an 18-hour co-culture. The altered expression of co-stimulatory molecules was mainly seen as an upregulation of CD83 and PD-L1, and a down-regulation of CD86 which could be indicative of a less activated phenotype. Interestingly, the same pattern could be replicated with the control 16HBE spheroid supernatants if the models were pre-incubated with IL-1b. Blockade of IL-1b have been shown to confer a degree of protection against developing lung cancer {R(96)and is therefore believed to have tumor promoting actions in the tumor microenvironment. A possible mechanism could be modulation of innate immune cells as a consequence of chronic inflammation, in part mediated by IL-1b.

6 CONCLUDING REMARKS

A central and recurring question following the discussion and presentation of results from the studies included in this thesis is:

What is the (potential) role of organotypic tissue models in medical research?

In my early exploration of organotypic tissue model, I was of the belief that diseases needed to be modelled in order to be studied comprehensively. Interrogating experimental setups by changing one parameter at the time in a disease setting seemed like the most natural and reasonable way to understand dynamic processes. I also felt that work related to human material/biopsies only described snapshots of a disease, and that heterogeneity within diseases would make it nearly impossible to *understand* the pathogenesis of a disease, and only provide descriptions on a limited number of situations.

The unprecedented evolution of big data research in all fields is slowly changing my mind. Although the research community is currently generating much more data than we could possibly handle, let alone make sense of, we are building a gold mine for future scientists to explore. The more data we can collect, the better – and it is only a matter of time before we have the tools to be able to properly organize and understand the collected data. Combining big data from biological material and behavioral patterns on an individual and societal level will enable creative comparisons and opportunities to ask specific questions that previously only was possible by reducing complexity and limiting variables to its extreme in biological/experimental models. The key difference between data generated from in vitro models and in vivo samples is that, except for the methodological limitations, the data generated from material collected from an individual are always ***true***. It can be an outlier or not a representative description of that particular disease, but the set of data is always true for that individual with the specific disease. In vitro data is only true for that specific experimental setup, which in best case scenario share features with its in vivo correlate.

If I were to guess on how organotypic tissue models would be used in the future, I would say it is in the pharmaceutical industry where screening for drugs requires more valuable output data than is currently the case. The field of toxicity screening have already implemented large-scale usage of models more complex than 2D cell cultures. 3D organotypic models could also be used as a screening tool that include not only the drug as variable parameter but incorporate the patient's own cells as part of the model and function as a variable in an effort to create personalized medicine. For organotypic tissue models to be useful in this context, validation of its tissue-resemblance is key. Organotypic tissue models could also potentially be used in transplantation settings, where it could provide a tissue-framework for proper regeneration/healing of damaged tissue, i.e. skin transplants. Organs that have the simplest, and most homogeneous histological organization are probably the best candidates for this type of use. Cornea, liver and pancreas could be some of the candidate organs, beside skin, that are worth trying to develop in vitro for transplantation purposes. The usefulness of organotypic tissue models as hypothesis-generating platforms remains to be proven. Today, it is likely too

laborious and inefficient for wide implementation in immunological research as the organotypic tissue models are far from not near perfect representation of real tissues. As I mentioned earlier, organotypic tissue models are not better or worse than any other model – but it is different compared to 2D culture systems. In some respect, the differences may be in favor of organotypic tissue models. In other instances, it complicates the interpretation and I believe it is fair to say that organotypic tissue models to date cannot be regarded as a "golden standard"-method to test any hypotheses specifically addressing disease pathogenesis, especially as it is unlikely to find comparable datasets to relate data generated using organotypic tissue models. Although, as a hypothesis-generating platform, I am more optimistic seeing a place for the use of organotypic tissue models.

Finally, I am hopeful that organotypic tissue models will play an increasingly larger role in medical research in the future and that the work in this thesis represents methodological proof-of-concepts which can be further explored with confidence once comprehensive validation and continued development of the model systems are further along.

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